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Loss of all or part of one copy of chromosome 17p is a frequent event in breast and ovarian tumors. We identified a common region of allelic loss between two highly polymorphic DNA markers on 17p13.3, YNH37.3 and YNZ22.1. These two markers are separated by less than 20 kbp. To date, only two genes have been reported that map within the critical region, OVCA1 and OVCA2. Both OVCA1 and OVCA2 are highly conserved evolutionarily. Western blotting and immunohistochemical approaches reveal that OVCA1 and OVCA2 is expressed in normal mammary and ovarian epithelium, and that their levels are significantly reduced or are undetectable in a high percentage of tumors and tumor cell lines. Somatic mutations are rare in OVCA1 and OVCA2, however, two germline missense mutations have been found in breast cancer-prone women who have tested negative for a BRCA1 or a BRCA2 mutation. Over-expression of OVCA1, but not OVCA2 appears to suppress breast and ovarian tumor cell growth in vitro. Screens for proteins that potentially interact with OVCA1 have uncovered a novel RNA binding protein, called BOV-1. Our goals are to evaluate the biochemical functions of OVCA1 and OVCA2 and determine their potential role(s) in breast and ovarian oncogenesis.

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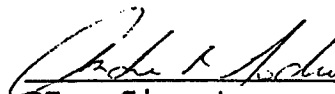
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INTRODUCTION:

Breast cancer is the second most common form of cancer in women, striking 1 out of 8 women in their lifetime. Ovarian cancer strikes fewer women but is generally at an advanced stage at the time of detection. Both diseases are controlled by multiple genetic defects, suggesting the involvement of many different genes, including tumor suppressors. According to the two-hit model of Knudson, both alleles encoding for a tumor suppressor must be lost or inactivated in order for cancer to develop. Based on this model, loss of heterozygosity (LOH) of alleles from tumor tissue has been used to suggest the presence of potential tumor suppressor genes.

The short arm of chromosome 17 is one of the most frequently altered regions in human breast and ovarian cancer. One locus of high allelic loss is at 17p13.1, and contains the tumor suppressor gene, *TP53*. However, we and others have shown a second region of LOH distal to the *TP53* gene, at 17p13.3, in breast tumors and ovarian tumors. Genomic abnormalities involving 17p13.3 has also been reported in primitive neuroectodermal tumors, carcinoma of the cervix uteri, medulloblastoma, osteosarcoma, astrocytoma (22), and acute myeloid leukemia and myleodysplastic syndromes, suggesting that a gene(s) on 17p13.3 may play a role in the development of a wide variety of neoplasms, including breast and ovarian cancer.

We have previously defined a minimum region of allelic loss (MRAL) on chromosome 17p13.3 in genomic DNA from ovarian tumors and breast tumors. Positional cloning and sequencing techniques revealed two genes in the MRAL, referred to as *OVCA1* and *OVCA2*, which overlap one another in the MRAL, and have one exon in common. Since translation of *OVCA1* does not proceed into the shared exon, the genes encode for completely distinct proteins. The function of OVCA1 and OVCA2 are unknown and their potential role in breast and ovarian oncogenesis has been a major focus of our studies. As requested, we have tried to focus this report on the progress we have made during the past 12 months that specifically relates to the research objectives listed in the approved Statement of Work (SOW) for year 3. We have also noted changes in our approach or progress made that are outside of the original goals stated in the SOW for year 4.

BODY:
Progress Report
Year 3

Months 25-30 Specific Aims 2; Complete any unfinished studies pertaining to Aim 2, and 3; Evaluate mutant forms of OVCA1 for altered growth suppressor function, analyze OVCA2 by western blotting for altered levels and/or molecular weights.

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To define the functional domains of OVCA1.

We have made six mutant constructs (**Figure 1**) and have introduced them into various cell lines (A2780, MCF-7, 293). All six mutant proteins are expressed as determined by Western blotting (**Figure 2**) and one has been shown to suppress colony outgrowth at the level seen with wild-type OVCA1. Stable cell lines expressing OVCA1Δ407 (which removes the last 36 amino acids at the carboxy terminus) show nearly identical rates of cell growth as those which express low levels of exogenous OVCA1, indicating that a portion of the C-terminus may not be important in the protein's function. In comparison, exogenous expression of the other mutants fails to alter the clonal outgrowth. It is not entirely clear if this lack of an observable phenotype of the other mutants is due to lower levels of expression of each or that the alteration(s) has completely inactivated the protein's ability to suppress cell growth. For example, the OVCA1Δ1-154 appears to be very unstable. The protein is expressed at low levels and appears as two discrete bands (**Figure 2**). In comparison, OVCA1Δ140, is expressed at high levels and fails to affect cell growth. We observed that removal of the putative transmembrane domain (amino acids 169 to 198; OVCA1Δ169-198) resulted in accumulation of the mutated protein primarily in the nucleus of MCF-7 cells (**Figure 3**). Our previous studies (both by cell fractionation and immunocytochemistry) have shown that OVCA1 is located in the cytoplasm as well as the nucleus. Overall, our results would indicate that both minor and major changes in the protein structure greatly affect its activity and that discrete functional domains may not exist. We are now refining our mutational analysis of OVCA1 to focus on more subtle changes (e.g., single base substitutions in exons and splicing variants that are listed in **Table 1** below). The mutant construct will still be of value regarding our proposed studies of proteins that interact with OVCA1.

To aid in our study of OVCA1-related growth suppression, we derived 293 cells that could rapidly upregulate OVCA1 levels several fold following exposure to Ponasterone A, an ecdysone analog (Figure 8 of the original proposal). We evaluated cell growth following OVCA1 induction and found that over-expression of OVCA1 failed to elicit a discernable phenotype. Based on these results, we hypothesized that 293 (an embryonic kidney cell line immortalized with adenovirus E1A) may not be responsive to exogenously expressed OVCA1. To determine if this observation was likely, we transfected parental 293 cells with OVCA1 expression vectors. Unlike A2780, MCF-7, MDA-MB8, SKBR3, HIO-118Nutu, and T47D tumor cells, 293's did not show reduced cloning efficiency following introduction of OVCA1 by transfection and G418 selection. Since 293 cells were immortalized with adenovirus E1A, we tested whether OVCA1 suppression was dependent on the retinoblastoma (RB) status of the cell. We obtained RB wild-type (U2OS) and mutant (Saos-2) osteosarcoma cell lines and found that expression of exogenous OVCA1 in Saos-2 failed to suppress growth. In comparison, U2OS cells appear to be as sensitive to the growth suppressive affects of OVCA1 as the other cell lines listed above. Of the 12 Saos-2/OVCA1 clones selected, 11 expressed high levels of OVCA1 as determined by Western blotting and grew at rates similar to the

parental cell line (data not shown). These results suggest that OVCA1 may function upstream of RB or that an intact RB pathway may be necessary for OVCA1 to exert its effect on cell growth.

To derive cells that are inducible and responsive to OVCA1, we transfected A2780 with the plasmid pVgRXR, which expresses the heterodimeric ecdysone receptor. The cells were then selected in Zeocin (0.2mg/ml) and fifty viable colonies were isolated. The clones were screened for ecdysone-inducibility by transfecting them with the plasmid pIND-lac Z, which expresses β-galactosidase only in the presence of an activated heterodimeric ecdysone receptor. Each transfection was done in duplicate. One of each pair of transfections had 10 μM of the ecdysone analog Ponasterone A added to the media at the time of transfection. Forty-eight hours after transfection and induction, the cells were fixed and then stained with X-GAL for β-galactosidase activity. Out of the fifty clones screened, two clones were found to express β-galactosidase in an ecdysone independent fashion, one clone was found to induce expression of β-galactosidase only weakly (blue X-GAL cleavage product was only apparent after 30 hours of staining), and two clones were found to induce detectable levels of β-galactosidase (blue X-GAL cleavage product was apparent after only 12 hours of staining). To further test the positive clones a liquid β-galactosidase activity assay was performed. The results of these assays were similar to that seen with the *in situ* β-galactosidase activity assay-two clones leaked expression of β-galactosidase, one clone induced expression only weakly (15% of maximum possible level), and two clones were able to induce reasonably high levels (80% and 75% of maximum possible levels) of expression. We are in the process of introducing the inducible OVCA1 construct. Cells will be selected, expanded, and tested as described for the 293 cells.

Mutational Analysis of OVCA1 and OVCA2 by SSCP.

Single-strand comformational polymorphism (SSCP) analysis was conducted on 75 ovarian tumors independent of LOH status for markers on 17p13.3, and 35 breast tumors demonstrating allelic loss for *OVCA1* and *OVCA2* and retention of *TP53*. Multiple sequence variants were identified throughout the gene (**Table 1**). These sequence variants were deemed to be polymorphisms, since these same alterations were either found in the corresponding germline or resulted in either conservative or silent amino acid substitutions. The frequency of these putative polymorphisms was determined by SSCP analysis of 100 chromosomes from control individuals (**Table 1**). Of note, we did detect single somatic mutations in introns 6, 10, and 11 when screening breast and ovarian tumors. All were single nucleotide substitutions and none were predicted to result in aberrant splicing of *OVCA1*.

We identified several alterations which suggested that aberrant splicing of *OVCA1* may be involved in carcinogenesis. A sequence alteration in intron 12 near the 5'-splice site was detected in four independent ovarian tumor cell lines, but not in the corresponding constitutional DNA for two of the individuals (the other two DNAs were not available). This variation has not been observed in the germline of our control

population. RT-PCR analysis of these tumor cell line RNA's revealed expression of an alternatively spliced transcript of *OVCA1*. However, the protein encoded by the modified transcripts is not predicted to be altered, therefore, it is not at present apparent how this acquired alteration contributes to ovarian cancer. The significance of this somatic mutation is under investigation.

Of potential interest, we identified two non-conservative amino acid substitutions, Ala34Asp and Arg389Ser. Each alteration was detected in the germline of a woman with breast cancer with a strong family history of the disease. In both cases the missense mutation/rare polymorphism was retained in the corresponding breast tumor DNA and showed reduction to homozygosity. Evaluation of more than 200 control chromosomes have failed to detect these sequence variants. The probands do not have unusual ancestries, indicating that the sequence alterations are unlikely to be related to a specific ethnic group. Unfortunately, we have not yet received the blood samples from additional family members. Both of these probands have tested negative for germline mutations in *BRCA1* and *BRCA2* (Godwin, unpublished data). Further studies are required to determine the functional consequences of these amino acid substitutions and to determine if these missense mutations/polymorphisms segregate with disease in the respective families. These mutations are being incorporated into OVCA1 and will be tested for alterations in the ability to suppress tumor cell growth *in vitro*.

Expression of OVCA1 in breast and ovarian tumors

OVCA1, either *in vitro* translated or bacterial expressed migrates as a 50 kDa protein. Western blot analysis of extracts prepared from several normal human tissues identified an 85 (p85), a 70 (p70), and the expected 50 (p50) kDa protein. Analysis of tumor extracts demonstrated the complete absence of p85 and p70 in all tumors examined and lost or reduced expression of p50 in extracts prepared from breast (18/46) and ovarian (21/59) tumors when compared to extracts from primary cultures of mammary or ovarian surface epithelial cells, respectively (Western blots not shown).

In order to further analyze the expression of OVCA1, immunohistochemistry was performed on normal ovaries and benign and malignant ovarian tumors using antibodies derived to the N- (TJ132) and C-termini (FC21 and FC22) of OVCA1. In epithelial cells of normal ovaries and ovarian tumors of low malignant potential, strong nuclear and cytoplasmic staining in the epithelial cells was observed using both antibodies (**Figure 4A & B** and data not shown). Interestingly, previous localization studies indicate that OVCA1 shuttles between the cytoplasm and nucleus in a cell cycle dependent manner (Bruening, unpublished data). In contrast to the normal ovaries and ovarian tumors of low malignant potential, 90% (9/10) ovarian adenocarcinomas showed little or no cytoplasmic staining using the N-terminal antibody (**Figure 5A**). However, varying intensities of nuclear staining (high/medium in 5/9 tumors and light staining in 4/9 tumors) was observed. Interestingly, no nuclear or cytoplasmic staining was observed with the C-terminal OVCA1 antibody in these sections (**Figure 5B**). Furthermore, there was no apparent correlation with cellular

proliferation (as determined by Ki-67 expression), BRCA1, and OVCA1 expression in these carcinomas (**Figure 5C & D**). These results suggest that in ovarian adenocarcinomas, OVCA1 may be undergoing an aberrant post-translational modification (reflected by a loss in immunoreactivity of the C-terminal antibody) which results in the mislocalization of OVCA1 to the nucleus and exclusion from the cytoplasm. Studies are underway to determine the molecular mechanisms regulating OVCA1 expression and localization in both normal and tumor tissue. Western blot analysis of these adenocarcinomas indeed shows that OVCA1 is expressed at the equivalent varying levels to those seen with the immunohistochemistry when probed with the N-terminal antibody. However the C-terminal antibody can also detect OVCA1 after western blot analysis, suggesting either that the post-translational modification is lost during the preparation of the extracts or that the conformation of the modified protein is masking the epitope in the paraffin sections even after the antigen retrieval process.

Expression of OVCA2 in breast and ovarian tumors

In the previous progress report we suggested OVCA2 was expressed as a 25 kDa protein and that it was potentially processed to a mature form (21 kDa). We had generated two polyclonal antibodies (Abs) against OVCA2. A MAP peptide corresponding to a.a. 176 to 190 of OVCA2 was used for the C-terminal Ab, and a KLH-conjugated peptide (a.a. 32 to 46 of OVCA2) was used for the N-terminal Ab. Both antibodies were immunoaffinity purified prior to use. To test the specificity of these antibodies Cos-1 cells were transfected with the genomic *OVCA2* expression vector under the control of a CMV promoter. These cells produced the predicted ~25 kDa protein, which could be detected with both a C-terminal and an N-terminal OVCA2 Ab. The same results were obtained when Cos-1 cells were transfected with a *HA-OVCA2* cDNA. Endogenous OVCA2 protein was detected in untransfected MCF-7 and MDA MB-468 extracts on Western blots probed with the N-terminal antibodies. However, endogenous OVCA2 protein could not be detected with the C-terminal Ab, due to a much lower affinity of the C-terminal Ab than the N-terminal Ab for OVCA2. When we began evaluating tumor samples, we found that the N-terminal antibodies also detected a major protein with a M.W. of ~21 kDa, while the C-terminal antibody did not. Through much investigation we found that the 21 kDa protein was a very abundant protein that was present in the blood serum and was not related to OVCA2. Therefore, three additional antibodies against OVCA2 have been produced: an N-terminal antibody raised against a peptide from amino acids 27-41 of OVCA2 (TJ143). Another N-terminal antibody raised against a peptide of amino acids 1-35 of OVCA2 (FC 24) and a C-terminal antibody raised against a peptide from amino acids 78-92 (TJ144). Western blot analysis of tumor extracts using these antibodies have demonstrated that OVCA2 levels are reduced in ~38% (9/24) of breast tumors and ~36% (8/22) of the ovarian tumors examined. OVCA1 and OVCA2 show a similar expression pattern whereby tumors with reduced levels of OVCA1 often showed reduced levels of OVCA2, which could be explained by haploinsufficiency at the *OVCA* locus whereby *OVCA1* and *OVCA2* are co-deleted.

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Immunohistochemistry with an anti HA-antibody, revealed that OVCA2 is primarily in the cytosol (data not shown). Subcellular fractionation studies have also shown OVCA2 predominantly in the cytosolic fraction. Immunohstochemistry, performed on normal ovaries, benign and malignant ovarian tumors using TJ143, demonstrated that OVCA2 was localized to the cytoplasm. Strong cytoplasmic staining was observed in the epithelial cells (surface epithelial and invaginations and inclusion cysts) of the 4 normal ovaries analyzed (**Figure 6A & B**). Strong cytoplasmic staining was also observed in 4 tumors of low malignant potential (**Figure 7A**). Varying levels of staining was observed in the tumors analyzed. Medium/high staining was seen in the 4 tumors of grade II/III and lower or no staining was seen in 5 tumors at grade III/IV (**Figure 7C**). All the sections stained positive for the epithelial maker cytokeratin (**Figure 7B & D**). Additional tissue sections are being evaluated, focusing on grade I ovarian tumors and breast tumors.

Months 31-36 Specific Aim 3; Complete site-directed mutation studies of OVCA1, isolate and sequence clones that bind OVCA1, initiate the characterization of these clones, and Future Aims if results warrant; Evaluate the role of OVCA1 in familial forms of breast and/or ovarian cancer and other types of cancer, construct OVCA2 expression vectors, establish cell lines expressing both OVCA1 and OVCA2.

Putative OVCA1 protein interactors.

We have established two baits for OVCA1, one including amino acids 2 to 161 (N-terminal bait) and another including amino acids 225 to 443 (C-terminal bait), and screened a human fetal brain cDNA expression library for proteins that potentially interact with OVCA1. We have completed the final round of evaluations in yeast for all but a few of the putative interactors. Of the N-terminal interactors, triosephosphate isomerase, progalanin, heat shock protein 86 and elongation factor 1-alpha (**Table 2a**) had shown some degree of specificity as OVCA1 interactors, but all failed to the high (HST) stringency tests.

The second interactor hunt using the C-terminal bait of OVCA1 (a.a 225 to 443) yielded ~60 clones with positive LEU/*LacZ* phenotype. **Table 2b** shows those clones to contain 23 different partial cDNAs, indicating some degree of redundancy (i.e. the number of independent isolates of the same cDNA). Sequence analysis of the 23 clones has identified several known and unknown proteins. The most redundant clone, #519, accounted alone for ~30% of the total cDNA isolated and passed both the low and high stringency tests. It represented 15 independent isolates of a cDNA of 745 bp in length, encoding for an ORF of 173 amino acids of an unknown protein. We refer to this gene as *BOV-1* (binding to OVCA1-1).

Interaction analysis: test of specificity.

In order to assess the specificity of interaction between OVCA1 (a.a. 225-443) and clone #519, two-hybrid assay for protein interaction was performed following standard protocols (Golemis and Serebriiskii, 1998). Plasmids expressing appropriate sets of LexA-fused protein, activation-domain (AD) fused proteins, and LexA operator LacZ reporter were cotransformed either into EGY48 or EGY191 yeast strains:

<u>LexA-fused proteins:</u> OVCA1 (a.a. 225-443), p53, CDK4, pRFHM1 (bicoid).

<u>AD- fused proteins:</u> BOV-1, pJG4-5 (empty vector).

<u>LexA operator LacZ reporters:</u> pMW107 (8 ops); pMW108 (2ops).

<u>LexA operator *LEU* reporters:</u> EGY48 (6 ops); EGY191 (2 ops).

To test the reproducibility, specificity and degree of interaction between OVCA1 (225-443) and clone #519, the interaction analysis was carried out under 2 different stringency conditions: low (EGY48/pMW107, more permissive reporters) and high (EGY191/pMW108, less permissive reporters). **Figure 8** shows the results obtained after analysis of interaction under high stringency conditions. For each pair of proteins, 10 independent colonies were tested in a set of 4 plates under non-induced (glucose –His,-Ura,-Trp)(plates A-C) or induced (galactose–His,-Ura,-Trp)(plates B-D) situation for either β-galactosidase activity (A-B), or growth on medium without leucine (C-D), which was scored over a period of 5 days. As shown in **Figure 8**, 10 out of 10 colonies expressing both the #519 and OVCA1 (a.a. 225-443) fusion proteins showed transcription activation of both LacZ (panel B) and LEU (panel D) reporter genes, but not when TP53, CDK4 or bicoid (pRHFM1) were used as bait. We also found that clone #519 failed to interact with OVCA2 and the N-terminal portion of OVCA1 (a.a. 2 to 161) (data not shown), demonstrating that clone #519 interacted strongly and specifically with the C-terminal portion of OVCA1 (a.a. 225-443), even under high stringency conditions.

Northern analysis of BOV-1.

The expression pattern of *BOV-1* mRNA was evaluated by multiple tissue Northern blotting. The random-primed 0.8-kbp #519 probe hybridized to three major mRNA species of ~5.5 kb, ~3.2 kb, ~1 kb (**Figure 9**). While these species are expressed in all tissues to varying degrees, the 1-kb transcript was most abundant in testis, heart, placenta, spleen, thymus, and lymphocytes (**Figure 9**).

Sequence Analysis.

Comparison analysis of clone #519 cDNA with the GeneBank databases demonstrated 100% nucleotide homology (E value 0.0) to a human EST from a breast tumor cDNA library. Further analysis by PSI-BLAST showed the predicted protein of clone #519 to have significant homology to the RNA binding domain (RBD) of a hypothetical protein in *C.elegans* encoded by cosmid R07E5.3 (E value 1.10^{-29}), and a *X.laevis* ribonucleoprotein (RNP) (E value 1.10^{-34}). Moreover, comparison of the #519 protein to the structure database (the Protein Database, PDB) indicated that #519 might be a member of either the small nuclear ribonucleoproteins (snRNP) or heterogeneous nuclear RNPs (hnRNP) (all E values $<1.10^{-12}$) protein families.

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All of the proteins in these families have RBDs, which are typically 88 to 101 amino acids long, and the folds of these proteins are all quite similar to one another. UP1, the N-terminal region of hnRNP A1 contains two RNA-recognition motifs (RRMs) in a single polypeptide chain, and its crystal structure has been recently revealed (Xu *et al.*, 1997). Analysis of the #519 RBD structure clearly indicates that residues 72-160 contain a single RBD, while 155-210 may contain a second RRM (**Figure 10**). Upon further sequence analysis, we observed that clone R07E5.3 was a composite sequence of two genes, which lie in close proximity in the *C. elegans* genome (Snf5 and an unknown gene). Therefore, clone #519 was found to be a highly conserved RNA binding protein and not related to Snf5 of the *C. elegans*.

Cloning of BOV-1.

To aid in the characterization of BOV-1, we isolated 30 cDNA clones from a human fetal brain library and sequenced a fourteen of them to determine the predicted amino acid sequence of BOV-1a, 1b, and 1c (**Figure 9**). Our results suggest that *BOV-1a* (the abundant 1-kb transcript) represents the entire coding region identified through the yeast two-hybrid screen (i.e., clone #519) and that *BOV-1b* and *1c* result from alternative exon splicing and use of multiple alternative polyadenylation signals (data not shown). The predicted proteins encoded by BOV-1b and 1c differ from BOV-1a in that the protein is likely to be 16 amino acids shorter (Figure 6; translation of protein encoded by *BOV-1b* and *1c* is predicted to start at the second methionine). We our currently mapping the chromosome location of *BOV-1* by fluorescent *in situ* hybridization (FISH) (Rao and Godwin). Like OVCA1, BOV-1 appears to be highly conserved evolutionarily (**Figure 11**). *C. elegans* encodes a protein with 60.6% identity and 75.4% similarity at the amino acid level, which makes it highly likely that the biochemical function of the BOV-1 homolog has been conserved from nematodes to humans.

Determine if BOV-1 interacts with OVCA1 under physiological conditions.

We have clearly demonstrated that OVCA1 and the protein encoded by clone #519 interact with high specificity using a yeast two-hybrid approach. Now that we have established the entire coding region for BOV-1, we will determine if it interacts with OVCA1 in mammalian cells by co-immunoprecipitation and co-localization approaches. Subcellular localization experiments in mammalian cells indicate that the OVCA1 protein is localized to punctate bodies, which are scattered throughout the cell and heavily clustered around the nucleus. The fact that OVCA1 (i.e., the 50 kDa form) has a primarily perinuclear localization gives support to the evidence presented here showing a strong and specific interaction between the C-terminus of OVCA1 and BOV-1, a putative ribonucleoprotein. In this aspect, polyclonal antibodies directed against BOV-1 are being derived. In the meantime, we have constructed a T7-tagged BOV-1 and a HA-tagged OVCA1 expression vector. Both have been co-expressed in 293 cells. As of yet, we have not been able to co-immunoprecipitate OVCA1 using T7 antibodies and BOV-1 using HA antibodies. If we are unable to demonstrate that both proteins interact by immunoprecipation of total protein extracts, we will try mixing

experiments with proteins purified from bacteria. These studies will aid in further determining the biological significance of this interaction in the context of how OVCA1 expression effects tumor cell growth.

Exogenous expression of OVCA2.

Growth suppression assays were carried out as described for OVCA1. Over-expression of full-length OVCA2 in MCF7 and A2780 cells resulted in no suppression of colony outgrowth as compared to vector-only controls (data not shown). Individual cell lines that expressed exogenous OVCA2 failed to show an alteration in growth rates in vitro as compared to parental cell lines. We have concluded that the growth suppression phenotype originally observed using cosmid clones that contained both OVCA1 and OVCA2 were the result of expression of OVCA1. Based on these results we did not attempt to derive cell lines that expressed both OVCA1 and OVCA2 as originally suggested.

Progress on Year 4.

Months 37-48. Specific Aim 3; Complete cloning and sequencing of clones that interact with OVCA1 or OVCA2, initiate characterization of function of these new proteins (if not already known), and Future Aims; Evaluate effect of OVCA2 expression alone or in combination with OVCA1 on morphology and cell growth, initiate mutational studies of the OVCA1 homolog in yeast, establish transgenic mice with regulatable OVCA1 expression vectors, initiate gene "knockout" studies in immortalized HOSE cells.

C. elegans Ovca1 knockouts

As indicated above, our major focus in regards to establishing the function of OVCA1 has been to determine if BOV-1 interacts with OVCA1 in mammalian cells. The other proteins we have identified using the two-yeast hybrid screen do not appear to interact as strongly as BOV-1. Therefore, our focus will be on establishing the interaction and determining the relevance of this interaction in terms of cell growth. We have turned to the *C. elegans* to help determine the normal function of OVCA1 and BOV-1. To help in these studies we have requested that a strain harboring a deletion of the C14B1.5b locus be derived through the *C. elegans* Knockout Program at the Sanger Centre (Cambridge, England). C14B1.5b (originally referred to as yky5) appears to be the *C. elegans* homolog of OVCA1. We have cloned and verified the genomic sequence for C14B1.5. Loss of function (LOF) C14B1.5b/Ovca1 worms will be evaluated for developmental abnormalities and sterility (due to abnormal cell proliferation). If a phenotype is evident in the LOF worms, then we will determine if human OVCA1 can functionally substitute for the nematode homolog. The request to the Sanger Centre was made nearly 8 months ago, yet we have not received the strain. As an alternative, in collaboration with Dr. Eric Moss (Associate Member, FCCC), we are deriving nematodes lacking *Ovca1* and/or *Bov-1* using a double-stranded RNA knockout approach. *C. elegans* cDNA clones have been obtained and double strand RNA is being prepared. Even though these studies in *C. elegans* are not a Specific Aim of our original proposal, we believe that this experimental system will complement our studies

and aid in uncovering the function of OVCA1. The value of such a strategy has been demonstrated in many cases, particularly in the studies of oncogenes, other tumor suppressors, and genes involved in programmed cell death.

Ovca1 and Ovca2 mouse knockouts

To also aid in our future studies of *OVCA1*, we have isolated a genomic clone for the mouse *Ovca1* and *Ovca2*. We have sequenced 7,510 bp of the mouse clone and identified the nucleotides corresponding to exons 4 through 13 of *OVCA1* and exons 1 and 2 of *OVCA2*. We have established a collaboration with Dr. Albert Wong (Thomas Jefferson Cancer Institute) to simultaneously knockout a portion of the *Ovca1* and *Ovca2* locus in mice. In case genomic deletion of *Ovca1/2* results in embryonic lethality, we have chosen the Cre-loxP recombination system to ultimately create *Ovca1/2* knockouts. The Cre protein from bacteriophage P1 is a site-specific recombinase that excises intervening DNA sequences located between two recombinase recognition sites (loxP) in direct orientation to each other. Therefore, we will create a construct in which exons 9 through 12 of *Ovca1* and exon 1 of *Ovca2* are flanked by the lox sites (flox). We chose to knockout this region initially because we have found that other genes may share some of these exons. We have identified by RACE methods several transcripts containing exons 1 and/or 2 of *OVCA2* in addition to several upstream exons of *OVCA1*. These transcripts code for different forms of OVCA2. An additional clone, referred to as *OVCA3* has also been uncovered. It contains a portion of exon 13 of *OVCA1* (or exon 2 of *OVCA2*) and several centromeric exons (proximal to *HIC-1*) (**Figure 12**). Sequence analysis of genomic *OVCA1/2* identifies multiple potential amino acid open reading frames that are not included in the original *OVCA1* transcript. To determine if these regions may represent express sequences, we made several DNA probes corresponding to these putative ORFs and hybridized them to multiple tissue Northern blots. We found that by using a DNA fragment that included exon 10 of *OVCA1* and several hundred bases of 5' and 3' flanking sequence, that a ~1.2 kb transcript was detected (**Figure 13**). This transcript, referred to as *OVCA5* is present at very high levels in testis and at low levels in brain, kidney, and pancreas (not shown). We had previously failed to detected *OVCA5* on northern blots using full-length *OVCA1* cDNA probes because it co-migrates with *OVCA2*. We are in the process of obtaining a full-length clone of *OVCA5*. Sequence analysis of partial clones and identification of polyadenylated clones indicate that *OVCA5* is transcribed in the opposite orientation as compared to *OVCA1* and *OVCA2* (**Figure 12**). Our studies suggest that the *OVCA* locus, which is highly conserved in mouse, may contain exons for at least 5 genes. By knocking out the region proposed, we should potentially affect all five genes. If a developmental phenotype is observed in the mouse, future studies will refine the region deleted to establish the culprit gene(s).

KEY RSEARCH ACCOMPLISHMENTS:
-Identified a protein (BOV-1) that interacts with OVCA1 in a yeast two-hybrid trap system.
-Demonstrated that BOV-1 is ubiquitously expressed.

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-Isolated numerous cDNA clones for BOV-1 and determined through sequence analysis that BOV-1 is a highly conserved RNA binding protein.

-Determined that several OVCA1 mutants fail to suppress tumor cell growth *in vitro*, indicating potential domains that may be of functional significance.

-Derived antibodies to OVCA1 (both N- and C-terminal) that work for Western blotting, immunohistochemistry and immunoprecipitation.

-Showed that OVCA1 levels are reduced in breast and ovarian tumors and that OVCA1 may be modified in some ovarian carcinomas as compared to normal ovarian epithelium and benign or LMP ovarian tumors.

-Derived antibodies to OVCA2 (both N- and C-terminal) that work for Western blotting, immunohistochemistry and immunoprecipitation.

-Derived inducible OVCA1 cell lines.

-Identified other genes within the *OVCA* locus.

-Cloned and sequenced the mouse *Ovca* locus.

REPORTABLE ACCOMPLISHMENTS (related to the tasks outlined in the approved SOW):

Schultz, D.C., Balasara, B., Testa, J.R., and Godwin, A.K. Cloning and localization of a human diphthamide biosynthesis-like protein gene, *hDPH2L2*. Genomics, 52:186-191, 1998.

Bruening, W. Prowse, A.H., Schultz, D.C., Holgado-Madruga, M., Wong, A., Godwin, A.K. Expression of OVCA1, a candidate tumor suppressor gene, is reduced in tumors and inhibits growth of ovarian cancer cells. (Submitted, Cancer Research, 1999).

Salicioni, A.M., Bruening, W., Vanderveer, L., Godwin, A.K. Functional characterization of OVCA1, a putative tumor suppressor. American Society of Human Genetics, submitted, 1999.

Prowse, A.H., Bruening, W., Godwin, A.K. OVCA1, and novel tumor suppressor, is aberrantly expressed in ovarian carcinomas. American Society of Human Genetics, submitted, 1999.

CONCLUSIONS:

In order for future therapies to be developed for the fight against cancer it is important to understand the basic molecular mechanisms that give rise to a specific cancer type. The fundamental mechanisms underlying the genetic basis of cancer are slowly being defined and involve alterations in genes which have been classified into three general categories: (i) protooncogenes are involved in growth promotion and the defects leading to cancer are a gain of function; (ii) tumor suppressor genes are negative regulators of growth and a loss of function gives rise to cancer; and (iii) DNA repair genes are involved in maintaining the fidelity of the genome and altered function can lead to increase rates of mutations in both classes of cancer-causing

genes. Cancer is a multistep process that involves alterations in many specific genes. The normal cell has multiple independent mechanisms that regulate its growth and differentiation and several separate events are required to override these control mechanisms. Progress is now being made in isolating these genes and the proteins they encode for, determining the normal cellular functions of the proteins and in investigating the mechanisms of tumorigenesis.

Breast cancer is a very common disease, causing about 10% of deaths in women in the Western World. Molecular genetic analysis of breast tumors has revealed many genetic aberrations that may represent important steps in tumor development. To understand the genetic pathways underlying breast tumor development, it is necessary to identify the genes affected by these genetic aberrations and establish any correlations between disruption of their function and tumor phenotype.

Chromosome 17 frequently shows loss of heterozygosity (LOH) in breast carcinomas. In addition, re-introduction of chromosome 17 fragments into breast cancer cell lines has been shown to suppress tumorigenicity. Therefore, inactivation of tumor suppressor genes on chromosome 17 appears to be a critical event in the pathogenesis of breast cancer. Although *TP53* at chromosome 17p13.1 is involved in the pathogenesis of breast cancer, LOH mapping studies in breast, ovarian and brain carcinomas have defined a region distal to *TP53*, at 17p13.3, thought to harbor a tumor suppressor gene. In addition, a fragment containing 17p13.3 have been shown to suppress the tumorigenicity of breast cancer cell lines. New genes, *OVCA1* and *OVCA2*, has been identified on chromosome 17p13.3, in this critical region of allelic loss. OVCA1 is composed of 12 coding and one non-coding exons, while *OVCA2* is composed of two exons: a unique exon 1, and an exon 2 which comprises part of the 3' untranslated region of *OVCA1*. Thus, the two genes are overlapping, but their protein products are completely distinct.

Much of our focus during the last year has been on trying to uncover clues about the function of OVCA1. We have found that OVCA1 is highly conserved and exists in two forms, a 50 and an 85 kDa protein. Evidence suggests that the 85 kDa form is encoded by an alternatively spliced form of *OVCA1*. $p50^{OVCA1}$ is localized to punctate bodies scattered throughout the cell but primarily clustered around the nucleus while $p85^{OVCA1}$ is found exclusively in the nucleus. Western blot analysis revealed that $p50^{OVCA1}$ levels are reduced or are absent in >30% of tumors examined when compared to extracts from normal cells and tissues, but $p85^{OVCA1}$ is rarely detected in tumors. Somatic mutations are rare in *OVCA1*; but two germline missense mutations have been found in breast cancer-prone women who have tested negative for a *BRCA1* or a *BRCA2* mutation. Attempts to create breast and ovarian cell lines that stably over-express the p50 form of OVCA1 have generally been unsuccessful. The clones that do express exogenous $p50^{OVCA1}$ do so at very low levels, and have dramatically reduced rates of proliferation, an increased proportion of the cells in the G_1 fraction of the cell cycle, and decreased levels of cyclin D, which may be caused by an accelerated

rate of cyclin D degradation (Bruening, W., *et al.*, submitted). Reversion of these cells to a more rapid growth phenotype is accompanied by complete loss of expression of exogenous OVCA1. Screens for proteins that potentially interact with OVCA1 have uncovered several known and some unidentified proteins, including a novel RNA binding protein (BOV-1) (Salicioni, A.M., *et al.*, in preparation). Studies are underway to further characterize the biochemical functions of this highly conserved, yet novel protein, and the proteins with which it interacts and determine the relevance of this interaction as it relates to normal and abnormal cell growth.

It is known that a multitude of RNA-binding proteins (RBPs) play key roles in the postranscriptional regulation of gene expression in eukaryotic cells. Once produced in the nucleus, mRNAs are transported to the cytoplasm where the protein synthesis machinery is located. All these processes are mediated by numerous RBPs and by small RNAs as stable RNP complexes. Characterization of these proteins had led to the identification of several conserved RNA-binding motifs, that have significant predictive value, and recent experiments have begun to illustrate how several of them bind RNA (reviewed by Siomi and Dreyfuss, 1997). The significance of these interactions is reflected in the recent discoveries that several human and other vertebrate genetic disorders (Dropcho and King, 1994; Buckanovich and Darnell, 1997; Nishiyama *et al.*, 1998) are caused by aberrant expression of RNA-binding proteins.

The RNA recognition motif (RRM) is the most widely found and best-characterized RNA-binding motif. It is composed of two short sequences, RNP1 and RNP2, and a number of mostly hydrophobic, conserved amino acids interspersed throughout the motif. Animal, plant, fungal and bacterial cells contain RNP motif proteins, which suggests that it is an ancient protein structure with important functions (Dreyfuss *et. al.*, 1993). The RRM is the only RNA-binding motif for which detailed structural information is available. The three-dimensional structures of the NH$_2$-terminal RBD of U1 snRNP A (Tang and Rosbash, 1996), the U1 domain of hnRNP A1 (Xu *et al.*, 1997) and the single RBD of hnRNP C (Gorlach *et al.*, 1994) have been determined and are very similar. Overall, RNP motif proteins have a modular structure reminiscent of transcription factors.
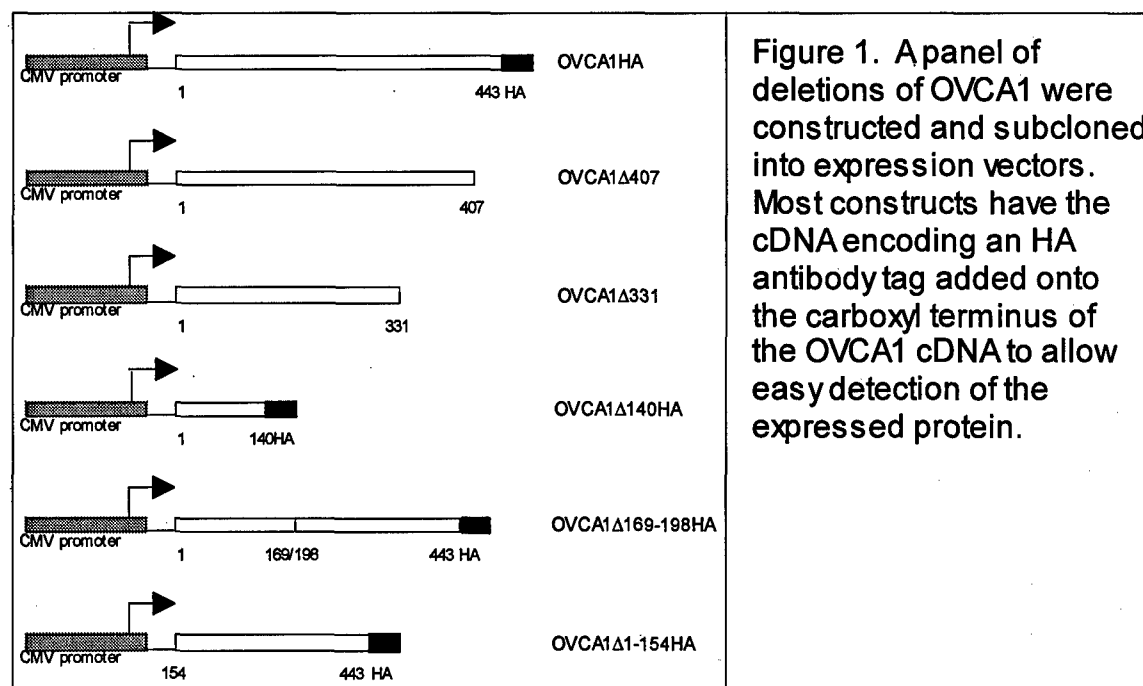
In particular, hnRNPs are abundant nuclear polypeptides, most likely involved in different steps of pre-mRNA processing. hnRNP A1, a prominent member of the hnRNP family, is one of the most abundant core proteins of hnRNP complexes in metazoan nuclei. It seems to act by modulating the RNA secondary structure and by antagonizing some splicing factors (SR proteins) in splice-site selection and exon skipping/inclusion. A role of A1 in the nucleo-cytoplasmic transport of RNA has also been proposed. These activities might depend not only on the RNA-binding properties of the protein but also on specific protein-protein interactions.

Our studies continue to suggest that OVCA1 have certain properties that are in common with a number of tumor suppressor genes. We have found that exogenous expression of OVCA1 can inhibit tumor cell growth and that expression of the protein is altered in both breast and ovarian tumors. Yet through our studies, we have not been able to establish a likely function for OVCA1. Therefore, we have initiated studies in *C. elegans* and mice to evaluate the effect(s) of altering OVCA1 (and in some cases OVCA2) expression on normal growth and development. By establishing such models, we should be better able to identify the function(s) of this very unique, but highly conserved protein.

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Dreyfuss G, Matunis MJ, Piñol-Roma S, Burd CG (1993). HnRNP proteins and the biogenesis of mRNA. *Ann Rev Biochem* 62:289-321.

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Xu RM, Jokhan L, Cheng X, Mayeda A, Krainer AR (1997). Crystal structure of human UP1, the domain of hnRNP A1 that contains two RNA-recognition motifs. *Structure* 5:559-570.
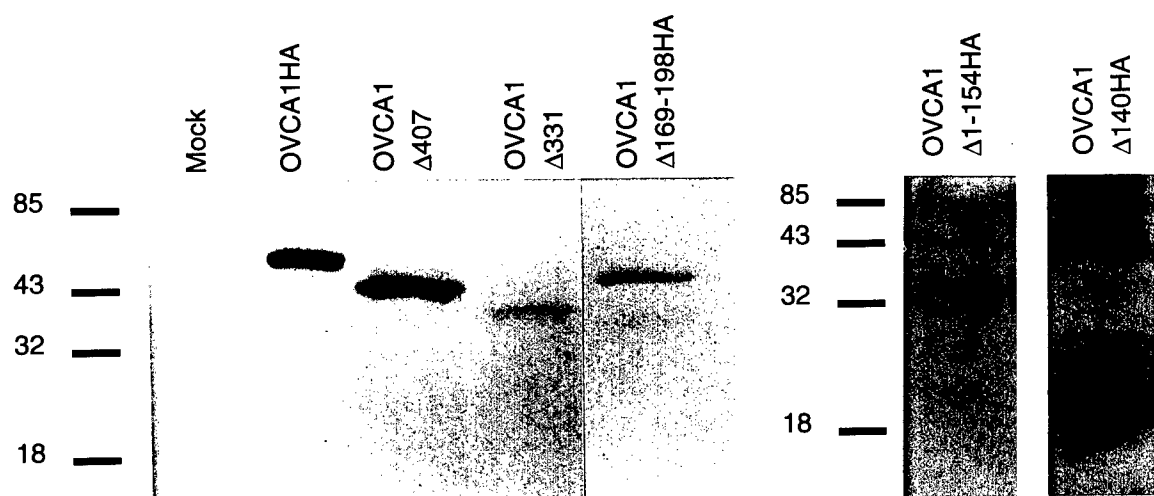
APPENDICES:

Figure 1. A panel of deletions of OVCA1 were constructed and subcloned into expression vectors. Most constructs have the cDNA encoding an HA antibody tag added onto the carboxyl terminus of the OVCA1 cDNA to allow easy detection of the expressed protein.

1

Figure 2. Western blot analysis of OVCA1 mutant proteins. COS-1 cells were transfected with the indicated CMV expression plasmids using Lipofectamine (Gibco BRL). Forty-eight hours after transfection, the cells were lysed in 2% SDS. 25 μg of each extract was separated by SDS-PAGE, transferred to Immobilon-P (Millipore), and probed with the indicated antibodies. The specific proteins were visualized by incubating with a donkey anti-rabbit antibody conjugated to horseradish. The left-hand panel was separated on 10% SDS-PAGE and probed with a specific anti-OVCA1 antibody TJ132, which recognizes an antigen located in the amino terminus of OVCA1. The right-hand panels were separated on 12% SDS-PAGE and probed with either an anti-HA tag antibody (OVCA1Δ1-154HA;Santa Cruz Biotechnology) or with TJ132 (OVCA1Δ140HA). OVCA1Δ1-154HA is expressed poorly and breaks down; the specific protein fragments are indicated with a bracket in the figure. The band seen at 43 kDa with the HA tag antibody is a cross-reacting protein endogenous to COS-1 cells.

Figure 3. Localization of OVCA1 deletion mutants by immunocytochemistry. MCF-7 cells were plated on coverslips and transfected with the indicated plasmids using Superfect. Forty-eight hours after transfection, the cells were fixed in 3.7% formaldehyde, dehydrated in methanol, rehydrated in TBS-Triton (50 mM Tris pH 8.0, 140 mM NaCl, 0.03% Triton-X100), and probed with the indicated antibodies. The signal was visualized by probing with a donkey anti-rabbit antibody conjugated to Texas Red (Jackson Immunoresearch Laboratories). The stained cells were observed on a Biorad MRC 600 laser scanning confocal microscope, using COMOS 7.0.1 software. The images were rendered and pseudo-colored with Voxel View 2.5.1 (Vital Images) software. Final prints were made using a codonics dye sublimation printer. Panels A, B, and C were probed with an anti-HA tag antibody (Santa Cruz Biotechnology), while Panel D was probed with the anti-OVCA1 antibody TJ132. Panel A was transfected with the plasmid CMV-OVCA1HA, which expresses full-length, wild-type OVCA1 plus a carboxyl terminal HA tag. Note the strongly stained punctate bodies near the nucleus superimposed on diffuse staining throughout the cytoplasm and some diffuse nuclear staining. Cells transfected with the plasmid CMV-Δ407, which removes the last 36 amino acids, had a similar staining pattern (data not shown). Panel B was transfected with the plasmid CMV-OVCA1Δ169-198HA, which expresses an HA-tagged OVCA1 protein with a deletion of the putative transmembrane domain. Note the lack of punctate bodies and the strong nuclear staining, plus some weaker diffuse cytoplasmic staining. Panel C was transfected with the plasmid CMV-Δ1-154HA, which expresses only the carboxyl terminus of OVCA1. Panel D was transfected with the plasmid CMV-OVCA1Δ140HA, which expresses only the amino terminus of OVCA1. Note in Panels C and D the lack of punctate bodies and the uniform diffuse staining throughout the cell.

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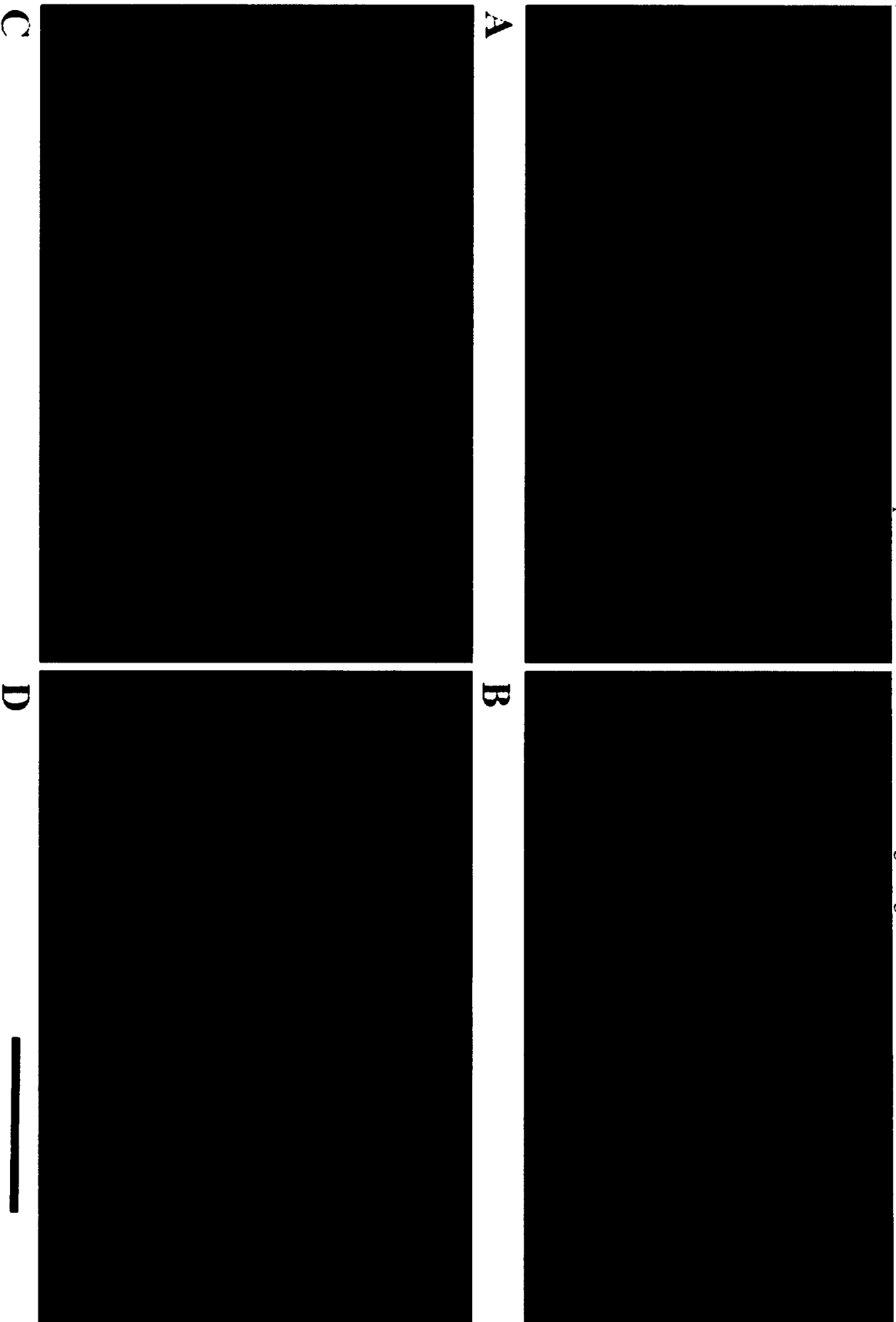
Figure 4. Immunohistochemical analysis of OVCA1 expression in ovarian epithelial cells. An ovary removed from a woman was fixed and embedded by standard methods. Sections were cut from the tissues and stained with the anti-OVCA1 (panels A & B), BRCA1 (panel C), and keratin (panel D). Shown are photomicrographs demonstrating expression of the various proteins in ovarian epithelial cells. In panel A the N-terminal OVCA1 (TJ132) antibody was used and in panel B the C-terminal OVCA1 (FC22) antibody was used.
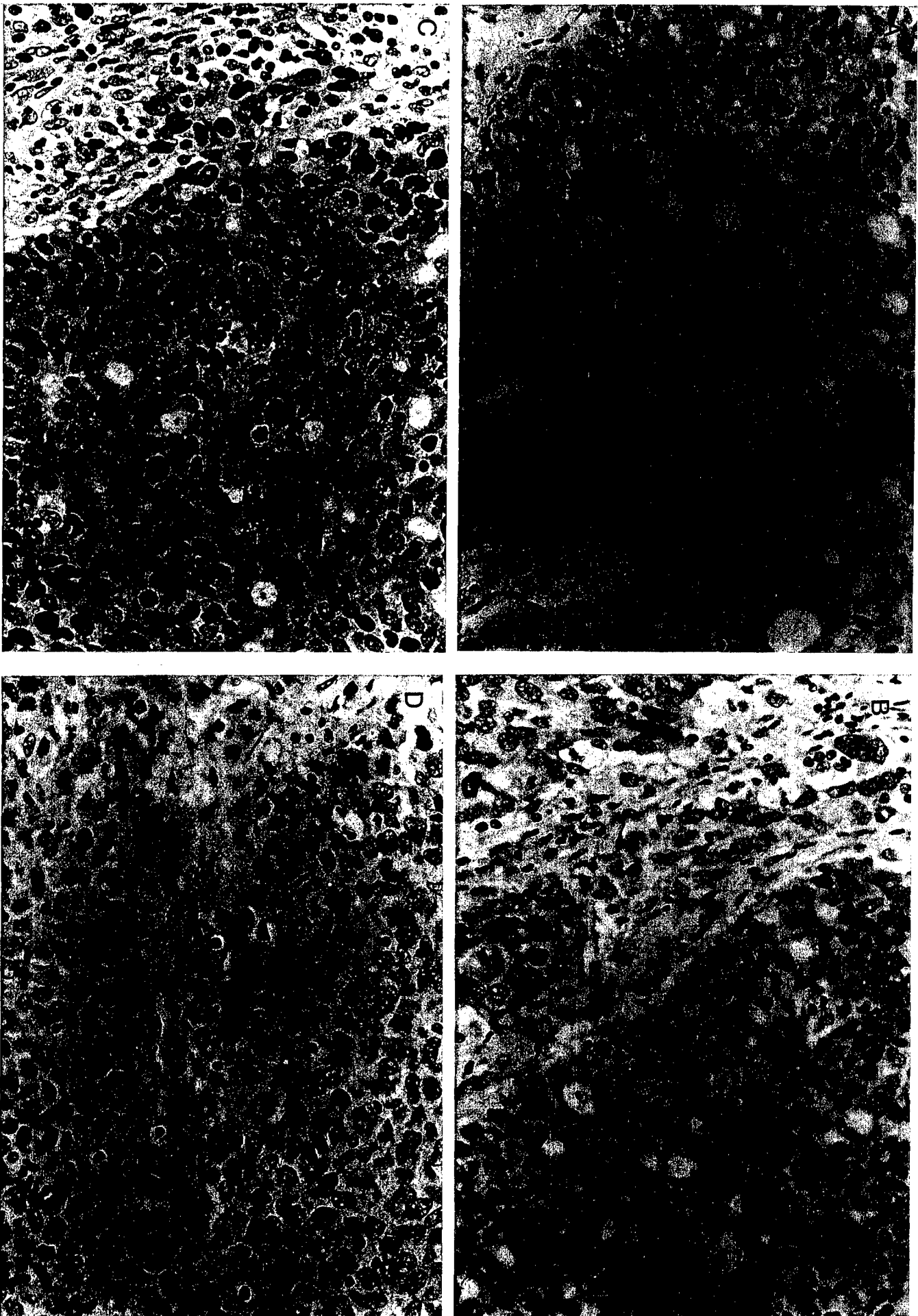
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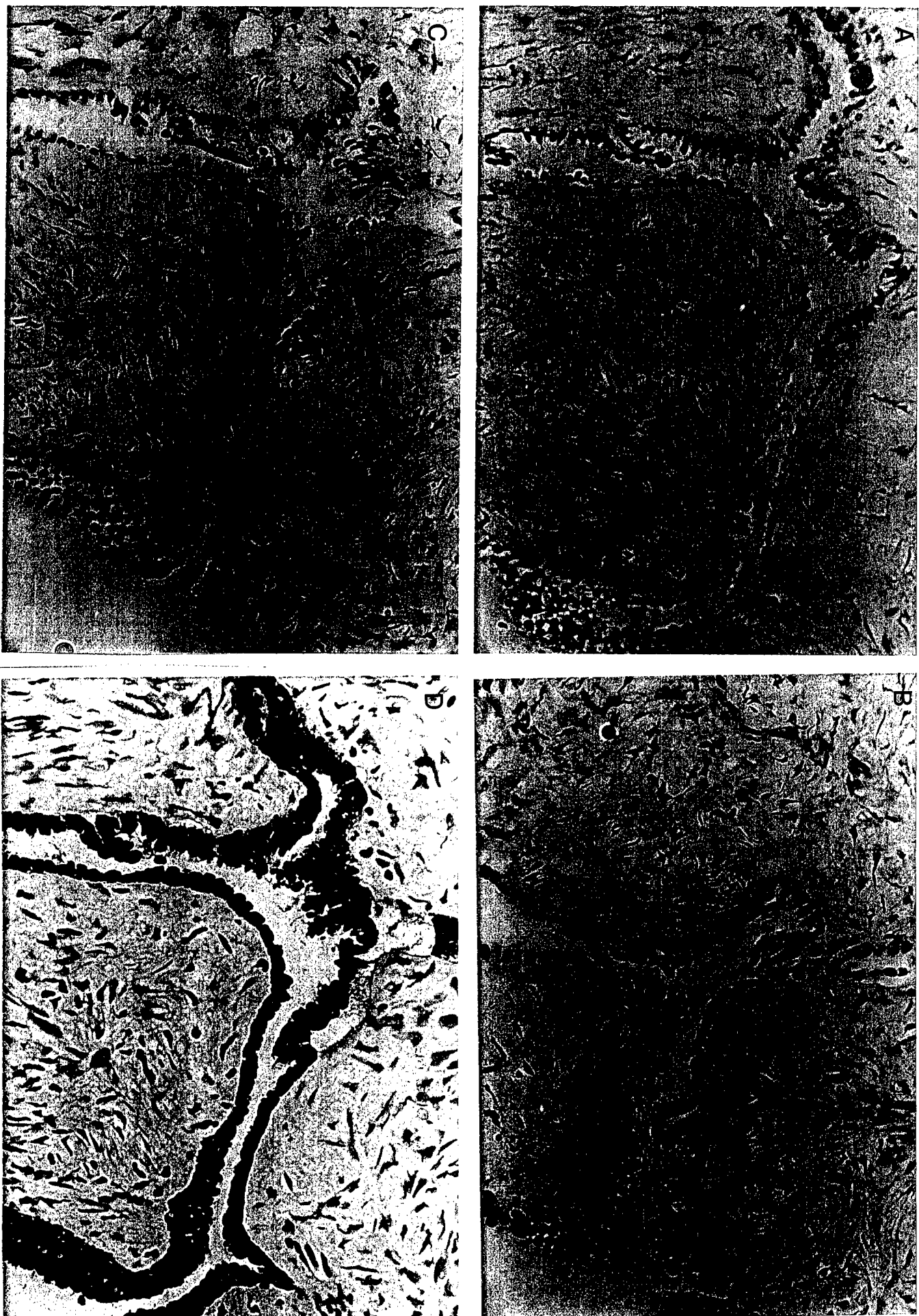
Figure 5. Immunohistochemical staining of OVCA1 in ovarian tumors. A papillary serous carcinoma of the ovary (grade III/IV) was removed, fixed and embedded by standard methods. Sections were cut from the tissue and stained with the anti-OVCA1 (panels A & B), mib-1/Ki-67 (panel C) and BRCA1 (panel D). Shown are photomicrographs demonstrating expression of the various proteins in the tumor. Panel A shows nuclear staining detected with the N-terminal OVCA1 (TJ132) antibody. Panel B shows a loss of immunoreactivity of the C-terminal (fiC22) OVCA1 antibody. Panel C shows that there are fewer Ki-67 positive cells than seen with the TJ132 antibody (Panel A) suggesting no apparent correlation between OVCA1 expression and cellular proliferation. Panel D shows that BRCA1 expression is also reduced in this tumor. In the panel of ovarian tumors examined we have observed no correlation between BRCA1 and OVCA1 expression (data not shown).
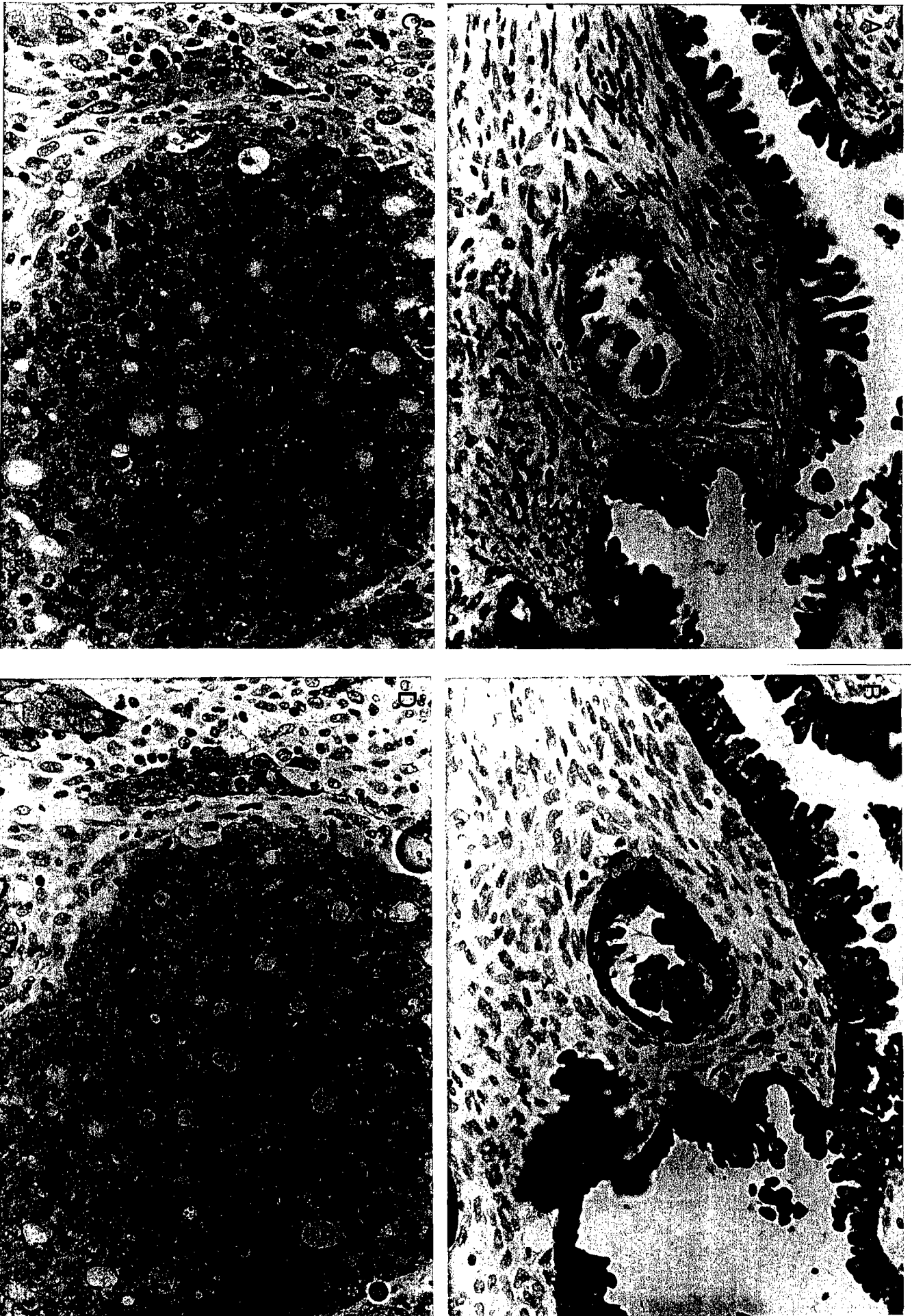
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Figure 6. Immunohistochemical staining of OVCA2 in ovarian surface epithelial cells. Sections were stained with N-terminal OVCA2 (TJ143) antibody. Panel A shows cytoplasmic staining of the surface epithelial cells of the ovary and Panel B shows cytoplasmic staining in ovarian epithelial cells lining a deep invagination.
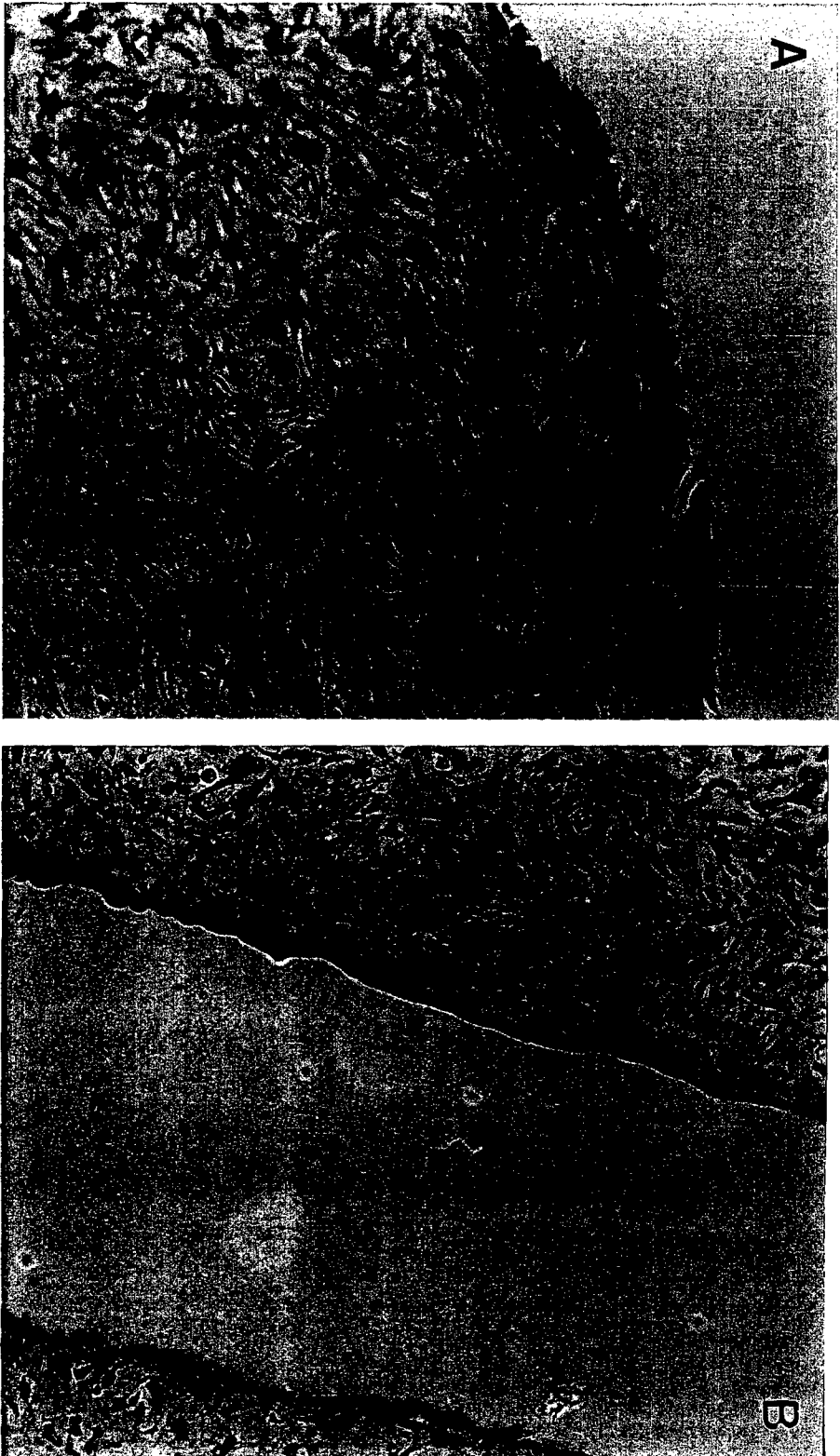
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Figure 7. Immunohistochemical staining of OVCA2 in ovarian tumors. Sections were stained with N-terminal OVCA2 (TJ143) antibody (Panels A & C) and with cytokeratin (Panels B & D). Panels A & B show expression of OVCA2 and cytokeratin, respectively, in a tumor of low malignant potential. Panels C & D show expression of OVCA2 and cytokeratin, respectively, in a papillary serous carcinoma (grade III/IV).
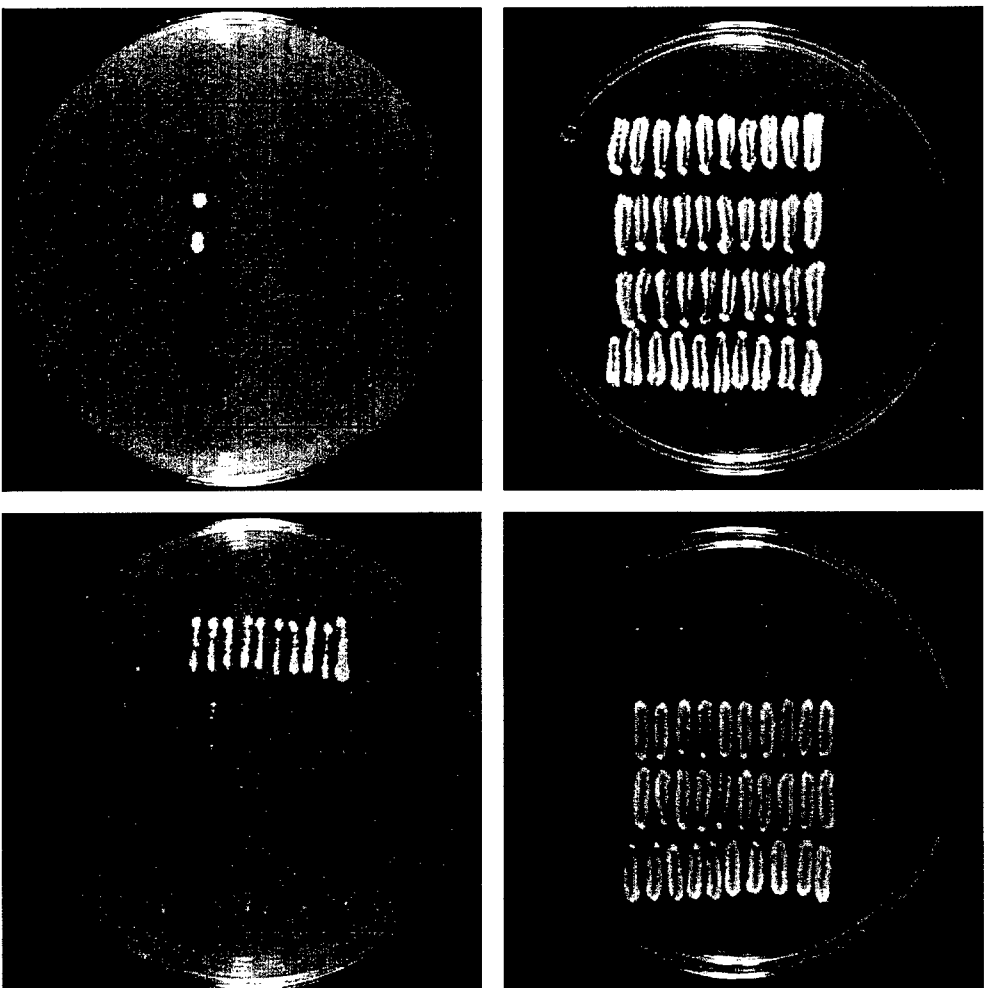
Figure 8. Specificity test for OVCA1 (225-443) and BOV-1 interaction under high stringency conditions (HST). Plasmids expressing BOV-1 protein and *lacZ* reporter pMW108 (containing 2 *lexA* operators) were co-transformed into *LEU* reporter EGY191 yeast strain (containing 2 *lexA* operators) which expressed one of the following LexA-fusion protein (columns from left to right): OVCA1 (225-443) (column '694'), pRFHM1, p53 or cdk4 (far right). For each pair of proteins, 10 independent colonies were tested in a set of 4 plates under non-induced (glucose; –His,–Ura,–Trp) (plates A-C) or induced (galactose; –His,–Ura,–Trp) (plates B-D) conditions for either β-galactosidase activity (A-B), or growth on medium without leucine (C-D), which was scored over a period of 5 days.

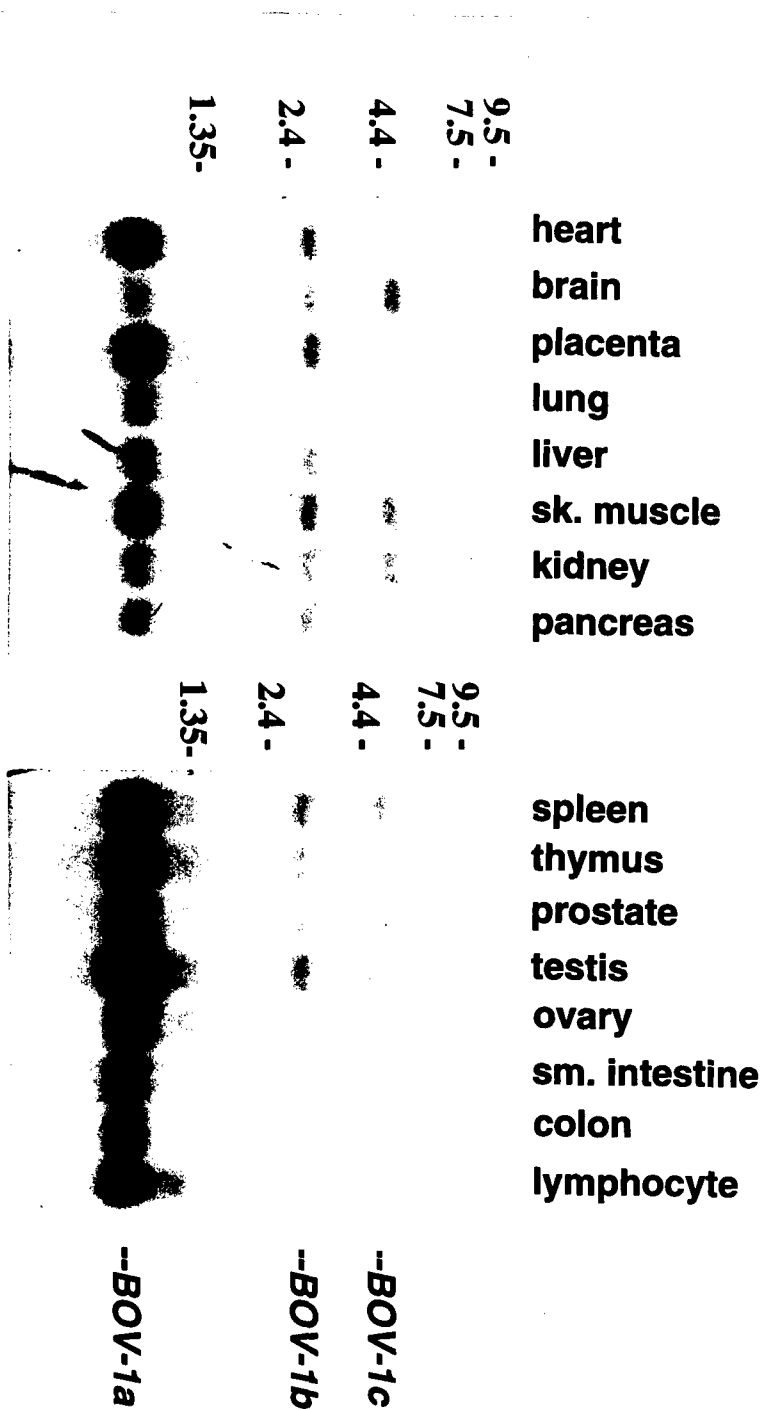8

Figure 9. Tissue expression pattern of BOV-1. Blots containing 5 µg of polyA⁺ selected mRNA from each of the indicated human tissues were hybridized with a 0.8 kbp cDNA clone of #519. Size standards are in kilobases.

Figure 10. Predicted RNA binding domains of clone #519 (i.e., BOV-1).

```
1              72                 158        221
--------------|-------------------|------------
partial RNA  full RNA domain   partial RNA
  domain                          domain
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bov-1      1 MADVLDHEAGGEDFAEDEDGDESHKGKEKAKKRRGRGFQSEGSRARMREDYDSVEQD-
R07E5.14   1 --------------MS-DNEVEMEDYVANAESRGRGLAQ--SRNRERITYDVVDEES
C23A1.09   1 -------------------------------------------------------
consensus  1                m  dd i l  a k grg  g   sr  r  yd ve  d

bov-1     60 -GDEPQPQRSVEGWIFVTGVHEEATEEDHKFAEYGEIKNIHLNLDRRTGYLKGYTLV
R07E5.14  42 SATSGAPQRSVEGWIFEVTNTHEEATEEDVHDKFEEYGKIKNIHLNLDRRTGILKGYALV
C23A1.09   1 ---MRPARSVEGRIIIVTGVHPEATEEQVMDLFASTGPVKNIHLNLDRRTGYMKGYALI
consensus 61 g  gPqrSVEGWilfVtgvHeEATEedvhDkfaeyG iKNiHlNLDRRTGylKGYalv

bov-1    119 EYETYKEAQAAMEGLNGQDLQPHSVDWCFVIGPPKg--RRRGRRRSPDRRRR--
R07E5.14 102 EYETQKEAHEAHDQSNDTDLLGQNVKVDWCFVRG----KKTSGKR--------
C23A1.09  57 EYATLHQAQKAVDEKN-LSLLDKLEVDAFEPERAPRPSISTRSQSPEVQHRDRD
consensus 121 EYeT keAq Amd N     dLlgq l VDwcFvrgp kg  kr sgrrrs spd rr

bov-1        ------
R07E5.14     ------
C23A1.09 116 VANAEP
consensus 181 VANAEP
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Figure 11. Alignment of BOV-1 predicted protein sequence with *C. elegans* R07E5.14 and *S. pombe* C23A1.09 hypothetical proteins. Conserved amino acid residues are shown in consensus. Identical residues are indicated by white letters in darkened boxes. Conserved residues are indicated by shading.

Figure 12. The *OVCA* locus and proximal flanking sequences on chromosome 17p13.3. The minimum region of allelic loss in breast and ovarian cancer is defined by D17S28 (YNH37.3) and D17S30/5 (YNZ22.1). Ten of the thirteen exons (4 through 13) of *OVCA1*, the promoter elements and both exons of *OVCA2* (alt*OVCA2*), and the single exon of *OVCA5* lie within this critical region. Arrows indicate the direction (telomere to centromere) and site of transcription initiation for *OVCA1*, *OVCA2*, and *HIC-1*. *OVCA1* has two first exons (1a and 1b). Also indicated are partial gene structures for *OVCA3* and *OVCA4*, and the *HIC-1* gene. E, *EcoRI*; H, *HindIII*; N, *NotI*.

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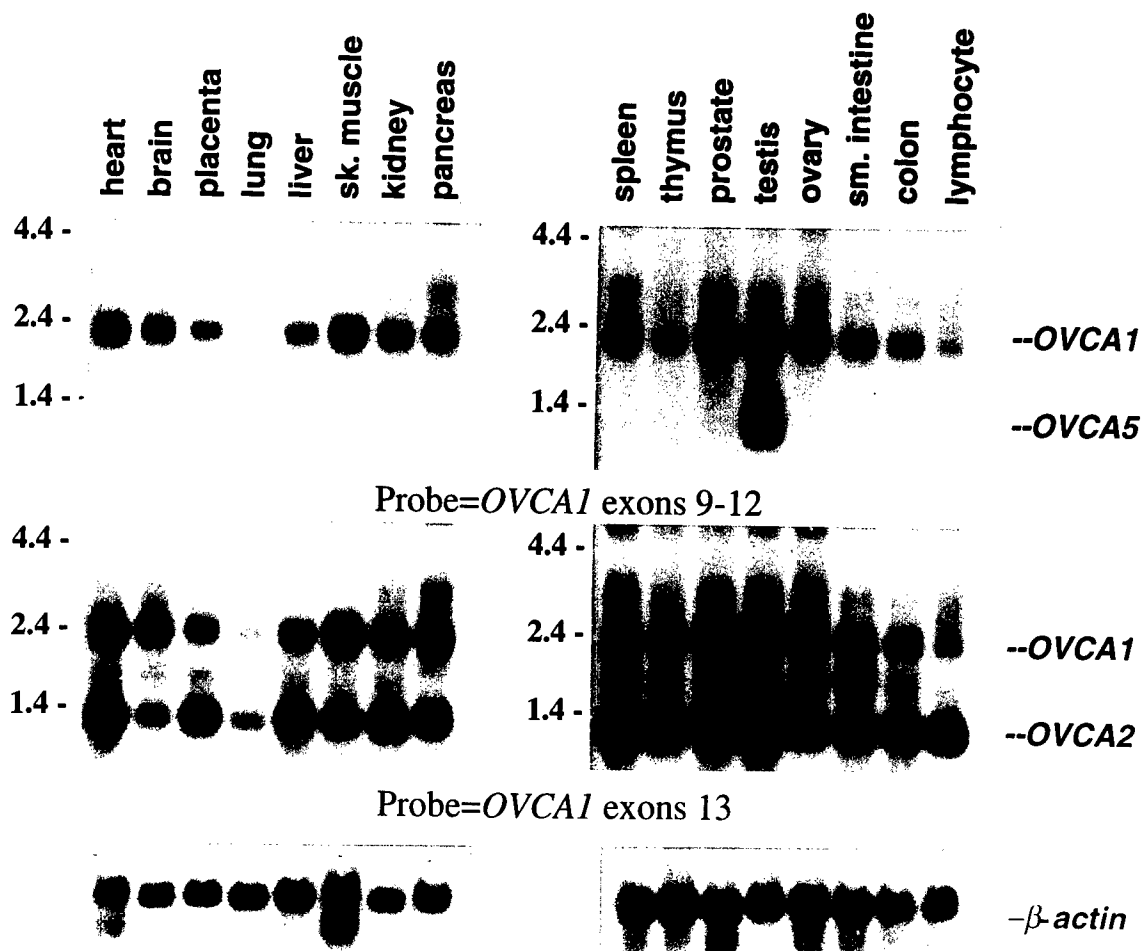**Figure 13.** Tissue expression pattern of *OVCA1*, 2, and 5. Blots containing 5 µg of polyA$^+$ selected mRNA from each of the indicated human tissues were hybridized with various probes (exons 9 through 12 or exon 13 of *OVCA1*). Size standards are in kilobases. Lower panel; blots were reprobed with a β-actin cDNA probe. Heart and skeletal muscle express two β-actin transcripts, 1.8-kb and 2.0-kb in size.

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Table 1. Nucleotide sequence variants observed in *OVCA1* in tumors

Exon	Codon	Base	Change	Result	Frequency[1]
1	7	2	C to T	Ala--Val	0.39
2	34	2	C to A	Ala--Asp	0.00[2]
2	72	3	C to T	Ala--Ala	ND
4	104	3	G to A	Val--Val	ND
4	138	﹐3	G to T	Leu--Leu	ND
5	188	3	G to A	Ser--Ser	0.20
IVS6			C to A		0.00
9	335	1	C to G	Leu--Val	0.09
9	337	3	C to T	Pro--Pro	0.18
IVS10			G to T		0.00
11	389	3	C to A	Ser--Arg	0.00[2]
IVS11			T to A		0.00
12	432	3	C to T	Ser--Ser	0.01
IVS12			G to A		0.00
IVS12			C to A		0.04
IVS12			del 4bp		0.01
13	NC		C to G		ND

Codon refers to the amino acid affected by the nucleotide change. Base indicates the nucleotide position of the codon affected. Change describes the nature of the nucleotide alteration. Result describes the affect the nucleotide alteration has on the amino acid.

[1]Allele frequency in control population was determined by examining 100 chromosomes from unaffected individuals.

[2]Allele frequency in control population was determined by examining 200 chromosomes from unaffected individuals

IVS, intervening sequences; ND; not determined; NC, non-coding sequence

Table 2a. OVCA1 (2-161 a.a.) interactors

Clone Number	Redundancy	cDNA encoded	Closest protein homology	Specificity tests [1]
2310	9	Triosephosphate-isomerase	Triosephosphate isomerase (human)	Positive LST
2359	8	Human mitochondrial tRNA	hypothetical 18K protein (goldfish)	Positive LST
2302	7	Heat shock protein (HSP) 86	HSP 86 protein (human)	Negative
2341	5	Progalanin	Galanin precursor (human)	Positive LST
2305	3	Cytoskeletal actin	Beta-actin (human)	Negative
2315	2	ADP-ribosylation factor 1	ADP-ribosylation factor 1 (3'-UTR)	Negative
2354	2	Thioredoxin peroxide reductase 2/pag	Thioredoxin peroxide reductase 2/pag (Human)	Negative
2327	1	Elongation factor (EF) 1-alpha	EF 1-alpha chain (human)	Negative
2337	1	Elongation factor (EF) Ts	Mitochondrial EF Ts precursor (human)	Negative
4516	1	chaperonin (HSP60)	Human HSP60	Negative
4533	1	Human cDNA EST (pregnant uterus)	Unknown	ND
2328	1	hPA28 proteosome activator	hPA28 proteosome activator subunit beta (human)	Negative
2314	1	ubiquitinol-cytochrome C reductase	ubiquitinol-cytochrome C reductase (RISP) precursor (human)	Negative
4544	1	Human cDNA EST (kidney)	Acyl carrier protein (human)	ND
4527	1	eukaryotic initiation factor 4AI	eukaryotic initiation factor 4AI (human)	Negative
4546	1	Human cDNA EST (adrenal gland)	P205 protein (receptor of activated protein) kinase C) (human)	ND
4545	1	c-myc binding protein	C-MYC binding protein MM-1	Negative

[1] Positive indicates the interactors passed the low (LST) or high (HST) stringency tests. Negative: the clone did not pass the specificity tests. ND= non-determined.

Table 2b. OVCA1 (225-443) interactors

clone number	redundancy	cDNA encoded	Closest protein homology	Specificity tests
519	15	Human cDNA EST (breast tumor tissue)	Unknown (RNA binding	Positive HST
218	8	Human homologue to rabbit endopeptidase	microsomal endopeptidase	Positive LST
305	4	Glucose-regulated protein (GRP78)	GRP78 protein (human)	Positive HST
430	4	Human cDNA EST (fetal heart)	unknown	Negative
342	3	Human cDNA EST (fetal heart)	Protein transport protein SEC61 (Mus musculus)	NR
205	2	Laminin-binding protein	Laminin receptor (human)	Negative
201	2	Voltage-dependent anion channel protein	Voltage-dependent anion channel protein (human)	ND
202	2	Human cDNA EST (total fetus)	unknown	Negative
212	2	Human cDNA EST (total fetus)	Beta-transducin protein (TRP-ASP rpts) (C.elegans)	NR
222	1	Prothymosin alpha	Prothymosin alpha protein (human)	NR
230	2	TAXREB107 DNA-binding protein[*]	Neoplasm-related C140 product (human thyroid carcinoma cells)	NR
321	2	Ribosomal protein L8[*]	60S ribosomal protein L8 (human)	NR
337	2	Beta-2-microglobulin	Beta-2-microglobulin precursor (human)	NR
209	1	Elongation factor 1-alpha	Elongation factor 1-alpha chain	Negative
221	1	p18/stathmin protein	p18/stathmin protein (human)	Negative
350	1	Thiol-specific antioxidant	Thioredoxin peroxidase 1 (human)	ND
363	1	Human cDNA EST (infant brain)	unknown	ND
369	1	Human cDNA EST (lung carcinoid)	Hypothetical protein (S. pombe)	ND
396	1	Pyruvate dehydrogenase kinase isoenzyme 2	Pyruvate dehyd. kinase isoenzyme 2 (human)	Negative
419	1	Human cDNA EST (fetal brain)	Cdc protein 48 homolog (M.jannaschi)	ND
514	1	Human cDNA EST (fetal liver spleen)	Phosphoryl. Regul. protein HP-10 (human)	Positive LST
540	1	Human cDNA EST (fetal liver)	C25 RNA polimerase III (S.cerevisiae)	ND
544	1	Human 26S protease S4 regul.subunit	26S protease regul.subunit 4 (human)	ND

[*]Likely to be a false positive. [1] Positive. The interactors passed the low (LST) or high (HST) stringency tests. Negative: the clone did not passed the specificity tests. NR: non-reproducible LacZ/LEU phenotype. ND= non-determined.

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MCMR-RMI-S (70-1y)   29 May 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request for Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has
reexamined the need for the limitation assigned to technical
reports written for Grant DAMD17-96-1-6088. Request the limited
distribution statements for Accession Documents Number ADB249637
and ADB275131 be changed to "Approved for public release;
distribution unlimited." These reports should be released to
the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at
DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management